



## ■ RESEARCH

# The protective role of nitric oxide-dependent innate immunosuppression in the early stage of cartilage damage in rats

## ROLE OF NITRIC OXIDE IN CARTILAGE DAMAGE

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## Objectives

Osteoarthritis (OA) is the most common form of arthritis, affecting approximately 15% of the human population. Recently, increased concentration of nitric oxide in serum and synovial fluid in patients with OA has been observed. However, the exact role of nitric oxide in the initiation of OA has not been elucidated. The aim of the present study was to investigate the role of nitric oxide in innate immune regulation during OA initiation in rats.

## Methods

Rat OA was induced by performing meniscectomy surgery while cartilage samples were collected 0, 7, and 14 days after surgery. Cartilage cytokine levels were determined by using enzyme-linked immunosorbent assay, while other proteins were assessed by using Western blot

## Results

In the time course of the study, nitric oxide was increased seven and 14 days after OA induction. Pro-inflammatory cytokines including tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6 were decreased. L-NG-Nitroarginine methyl ester (L-NAME, a non-specific nitric oxide synthase inhibitor) significantly decreased cartilage nitric oxide and blocked immune suppression. Further, L-NAME decreased Matrix metalloproteinase (MMPs) and increased tissue inhibitor of metalloproteinase (TIMP) expression in meniscectomised rats.

## Conclusion

Nitric oxide-dependent innate immune suppression protects cartilage from damage in the early stages of OA initiation in rats.

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## Article focus

- The role of nitric oxide (NO) in osteoarthritis (OA) initiation in rats.
- Relationship between nitric oxide and cytokine releases in osteoarthritis.

## Key messages

- Cytokine production in cartilage was decreased in OA initiation.
- Inhibiting NO generation increased cytokine but decreased matrix metalloproteinase levels

## Strengths and limitations

- Examining the role of nitric oxide in the very early stage of cartilage damage in OA.

- The randomised design with comparative control group.
- It was a small animal experimental OA model, large animal study should be conducted in the future.

## Introduction

Osteoarthritis (OA) is the most common form of arthritis, affecting approximately 15% of the human population.<sup>1</sup> Due to its predilection for lower extremity joints such as the knee and hip, OA is the leading cause of lower extremity disability amongst older adults with an estimated lifetime risk for knee OA being approximately 40% in men and 47% in women.<sup>2</sup> OA is characterised by chronic degeneration of joint structures in

the body including the synovial membrane, cartilage, and bone tissues.<sup>3</sup>

The classic characteristic of this disease is the degradation of cartilage, a hallmark of OA, in the joint space leading to swollen and 'creaky' joints. Although trauma is perhaps the first event in OA development, the host inflammatory response plays an important role in the pathogenesis of OA, and inflammation is believed to be the major factor in symptomatic OA.<sup>4,5</sup> However, the mechanism of cartilage degradation remains unclear.

Matrix metalloproteinase (MMP) has been reported to play a crucial role in regulating cartilage breakdown.<sup>6</sup> The concentration of MMPs in synovial fluid and cartilage in osteoarthritis patients is elevated, which is positively associated with the severity of osteoarthritis.<sup>7-9</sup> Further, tissue inhibitors of metalloproteinases (TIMPs) act in the tissue environment to neutralise used proteinases, thereby preventing excessive and unwanted degradation away from the sites of metalloproteinase production.

Nitric oxide (NO) is a predominant mediator in progression of OA and chondrocyte apoptosis.<sup>10,11</sup> A high degree of NO in the serum and cartilage of patients with arthritis has been shown in relation to normal cases.<sup>12,13</sup> Increased NO produced by inducible NO synthase (iNOS) is an important catabolic element in the activation of tumour necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$ .<sup>14</sup> Immunohistochemical analyses have confirmed the existence of iNOS in OA synoviocytes and chondrocytes.<sup>15,16</sup> Previous studies indicated that increased concentration of nitrite (a NO metabolite) in serum and synovial fluid samples of patients with rheumatoid arthritis and OA and have therefore suggested a role for NO as an inflammatory mediator in rheumatic diseases.<sup>15,16</sup> However, the exact mechanism in which NO regulates the immune system in OA chondrocytes remains unclear. The aim of the present study was to investigate the role of cartilage NO in innate immune regulation and the initiation of OA in rats.

## Materials and Methods

**Animals.** A total of 45 male SPF Sprague Dawley rats weighing 200 g to 300 g were obtained from Laboratory Animal Center, National Cheng Kung University, Tainan, Taiwan. They were individually housed in a room with a 12-hour dark/light cycle and central air conditioning (25°C, 70% humidity), were allowed free access to tap water, and fed a standard rodent diet from Richmond Standard PMI Feeds, Inc (St Louis, Missouri), with or without a sesame oil supplement. The animal care and experimental protocols were followed in accordance with nationally approved guidelines.

**Experimental designs.** Experiment I was a time course study of meniscectomy-induced cartilage NO and pro-inflammatory cytokine production in rats. Rats were divided into two groups of fifteen. Group I (Sham group),

rats received the sham operation only; and Group II (OA group) rats received a meniscectomy operation only. Cartilage nitrite (a marker for NO production) and pro-inflammatory cytokine including TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were assessed 0, 7, and 14 days after the sham or meniscectomy operation.

Experiment II considered the role of NO in cartilage pro-inflammatory cytokine, MMPs, and TIMP levels. Rats were divided into three groups of five. Group I (sham group), rats received sham operation only; Group II (OA group), rats received meniscectomy operation only; and Group III (OAL group), rats received meniscectomy operation and L-NG-Nitroarginine methyl ester (L - NAME, a non-specific NO synthase inhibitor) (20 mg/kg/d for 14 days, orally). Cartilage sample were collected 14 days after meniscectomy from Groups I, II, and III, respectively. OA induction

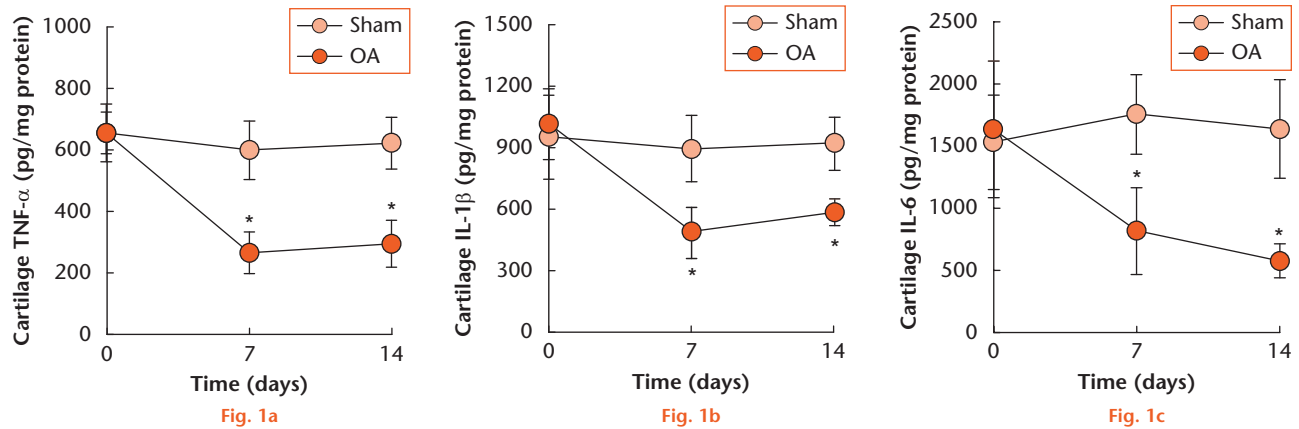
Meniscectomy surgery was performed under 3.5% isoflurane inhalational anesthetics.

All rats received cephalexin (Ceporex oral drops) (0.03 ml/100 g body weight) one hour pre- and 12, 24, and 36 hours post-operatively. A small incision was made longitudinally down the medial side of the knee and a cauteriser was used to work through both the connective tissue and muscle layers until the medial collateral ligament, anchoring the medial meniscus to the tibial plateau, was identified. The ligament was grasped at the tibial end and cut until fully transected. The ligament was then transected again at the femoral end to remove the portion overlying the meniscus, which was freed from the fine connective tissue, allowing a full thickness medial meniscal transection. Sham animals underwent the same surgical procedure with the omission of medial meniscal transection.<sup>3</sup>

**Protein concentration in cartilage.** Rat articular cartilage was collected after the rats were killed. We homogenised collected cartilage samples in ice-cold Milli-Q (MQ) water (1:10 w/v) with a tissue homogeniser. After centrifugation at 3000 g for ten minutes, supernatant was collected for protein assay. The protein concentration in tissue homogenate was determined by using protein assay dye (Bio-Rad Laboratories, Hercules, California) according to the commercial protocol.

**NO concentration in cartilage.** We homogenised collected cartilage samples in ice-cold MQ water (1:10 w/v) with a tissue homogeniser. After centrifugation at 3000 g for ten minutes, supernatant was collected. The amounts of nitrite in cartilage were measured following the Griess reaction by incubating 100  $\mu$ l of tissue homogenate with 100  $\mu$ l of Griess solution at room temperature for 20 minutes. The absorbance was measured at 550 nm by a spectrophotometer.<sup>17</sup> Nitrite concentration was calculated by comparison with a standard solution of known sodium nitrite concentrations.

**TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 levels in cartilage.** To examine the role of NO in innate immunosuppression, cytokines



Time course study of meniscectomy-induced cartilage nitric oxide production in rats. Rats were divided into two groups of five. Group I (Sham group), rats received the sham operation only; and Group II (OA group) rats received the meniscectomy operation only. Cartilage nitrite (a marker for nitric oxide production) was assessed 0, 7, and 14 days after the sham or meniscectomy operation. Data are expressed as means and standard deviation. \* $p < 0.05$  compared with Sham group (Group I).

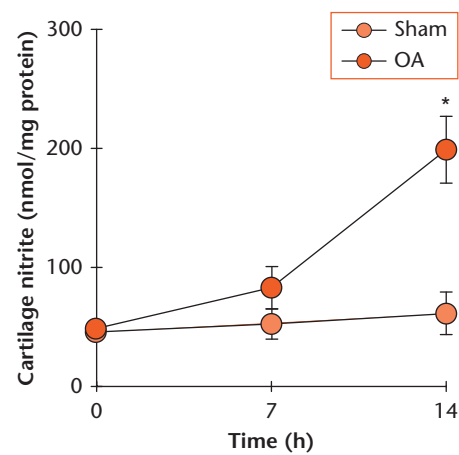
(the commonly used biomarker of immune response)<sup>18</sup> including TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in cartilage were determined. TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 levels were quantitatively measured by using an ELISA kit (Duo-Set; R&D Systems Inc., Minneapolis, Minnesota). Briefly, the sample was incubated with biotinylated rabbit antibody for two hours, after which streptavidine-conjugated horseradish peroxidase was added for 20 minutes. The peroxidase reaction was initiated by adding 3,3',5,5'-tetramethylbenzidine/H<sub>2</sub>O<sub>2</sub> (R&D Systems) for 30 minutes, and then stopped by adding 0.5 M H<sub>2</sub>SO<sub>4</sub>. The absorbance was measured at 450 nm.

**Western blotting.** A 50-microgram protein was loaded on SDS-PAGE, and then transferred to nitrocellulose sheets (NEN Life Science Products, Inc., Boston, Massachusetts). After blocking, the blots were incubated with MMP-2, MMP-9, TIMP-2, or  $\beta$ -actin antibody (Abcam, Cambridge, United Kingdom) (dilution 1:1000) in 5% non-fat skimmed milk (using  $\beta$ -actin as a loading control). After the blots were washed, they were incubated with secondary antibodies conjugated with alkaline phosphatase (dilution 1:3000) (Jackson ImmunoResearch Laboratories Inc., Philadelphia, Pennsylvania). Immunoblots were developed using bromochloroindolyl phosphate/nitroblue tetrazolium solution (Kirkegaard and Perry Laboratories Inc., Baltimore, Maryland).

**Statistical analysis.** Data were expressed as mean and standard deviation (SD). One-way analysis of variance (ANOVA) followed by student's *t*-test analysis was used to make pairwise comparisons between the groups. Statistical significance was set at  $p < 0.05$ .

## Results

**The time course of cartilage NO production and immune response in meniscectomy-induced OA.** To examine the roles of NO and the immune response in the initiation of OA, cartilage nitrite and cytokines were assessed.



Time course study of meniscectomy-induced cartilage pro-inflammatory cytokine production in rats. Rats were divided into two groups of five. Group I (Sham group), rats received the sham operation only; and Group II (OA group) rats received the meniscectomy operation only. Cartilage pro-inflammatory cytokines including TNF- $\alpha$  (A), IL-1 $\beta$  (B), and IL-6 (C) were assessed at 0, 7, and 14 days after sham or meniscectomy operation. Data are expressed as means and standard deviation. \* $p < 0.05$  compared with Sham group (Group I).

Cartilage nitric levels in OA groups were significantly increased compared with that in the sham groups at day 14 after meniscectomy surgery (Fig. 2). However, cartilage TNF- $\alpha$  (Fig. 1a), IL-1 $\beta$  (Fig. 1b), and IL-6 (Fig. 1c) levels in the OA group were significantly lower compared with that of the sham group at post-operative days 7 and 14.

**Role of NO in immune response regulation in meniscectomy induced OA.** To examine the role of NO in immune response regulation, the concentration of cartilage nitrite, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were determined. Cartilage NO production in the OA group increased compared with that of the sham group, while L-NAME significantly decreased NO compared with the OA group (Fig. 3). In

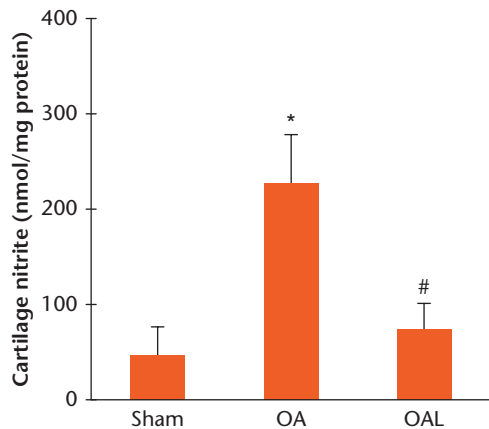


Fig. 3

The effect of L-NAME on cartilage nitric oxide production in rats. Rats were divided into three groups of five. Group I (sham group), rats received the sham operation only; Group II (OA group), rats received the meniscectomy operation only; and Group III (OAL group), rats received the meniscectomy operation and L-NAME (20 mg/kg/d for 14 days, orally). Cartilage nitrite levels were determined 14 days after meniscectomy from each group, respectively. Data are expressed as means and standard deviation \* $p < 0.05$  compared with sham group (Group I). # $p < 0.05$  compared with OA group (Group II).

addition, although the levels of TNF- $\alpha$  (Fig. 4a), IL-1 $\beta$  (Fig. 4b), and IL-6 (Fig. 4c) were significantly decreased in both OA groups, L-NAME treatment maintained all measured cytokine levels in OAL groups (Fig. 4).

**Role of NO in regulating cartilage breakdown in meniscectomy induced OA.** To examine the role of NO in regulating cartilage breakdown, we assessed the active form MMP-2 and MMP-9, as well as TIMP-2 expression in cartilage. Both cartilage MMP-2 and MMP-9 expression were decreased in OA group compared with the sham group. However, MMP-2 and MMP-9 was significantly higher in the OAL group compared with that in the OA group (Fig. 5). Furthermore, L-NAME significantly reversed the increase of cartilage TIMP-2 expression. The TIMP-2 level in the OAL group showed no difference compared with that in the sham groups (Fig. 6).

## Discussion

In the present study, we have illustrated the role of NO in the initiation stage of cartilage damage in rats. Inhibiting cartilage NO synthesis blocked immune suppression, as well as decreased MMPs and increased TIMP expression in meniscectomised rats. We suggest that NO plays a protective role in the very early stage of cartilage breakdown and subsequent development of OA.

Accumulation of NO may be associated with immune suppression in rat cartilage after meniscectomy. Although NO has been regarded as a pro-inflammatory mediator, its role in regulating the immune response is still paradoxical.<sup>19</sup> Some studies have indicated that NO enhances the immune and inflammatory responses;<sup>20,21</sup> however, other studies have shown that NO suppresses immune response and inflammation.<sup>22,23</sup> In the present study, more NO accumulation and less immune response was found in cartilage in the early stage of the experimental rat OA model. Furthermore, inhibiting NOS activity by L-NAME blocked the immune suppression. It is likely that the accumulation of NO may be involved in the suppression of the innate immune response in the early stage of cartilage damage.

NO-associated innate immune suppression may play a beneficial role in maintaining cartilage integrity in the early stages of OA. Articular cartilage is considered to be 'immune privileged'<sup>24</sup>, meaning it is able to tolerate the introduction of antigens without eliciting an inflammatory immune response, in that transplanted cartilage from unrelated donors does not elicit immune rejection responses.<sup>25,26</sup> Previous studies have indicated that prostaglandin (PG) E2 mediates the immune suppression in cartilage.<sup>27,28</sup> Inhibiting PGE2 by using non-steroidal anti-inflammatory drugs (NSAIDs) accelerated cartilage breakdown in experimental and clinical studies.<sup>29,30</sup> Moreover, one of the recent clinical trials of potent anti-inflammatory therapies, including the use of systemic and intra-articular biological agents to inhibit TNF $\alpha$  and IL-1 $\beta$

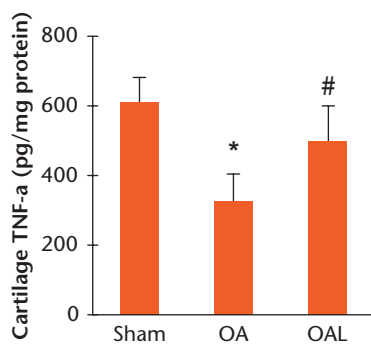


Fig. 4a

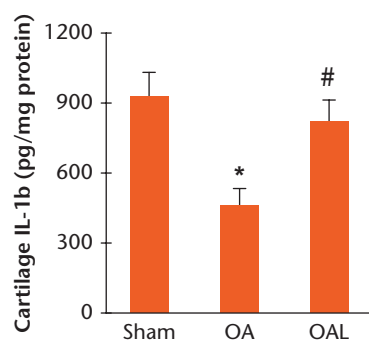


Fig. 4b

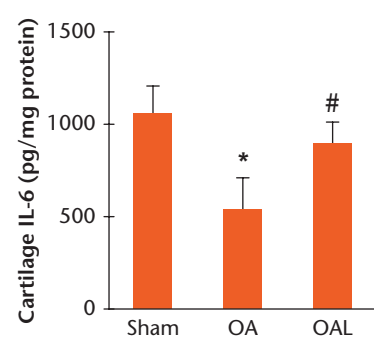
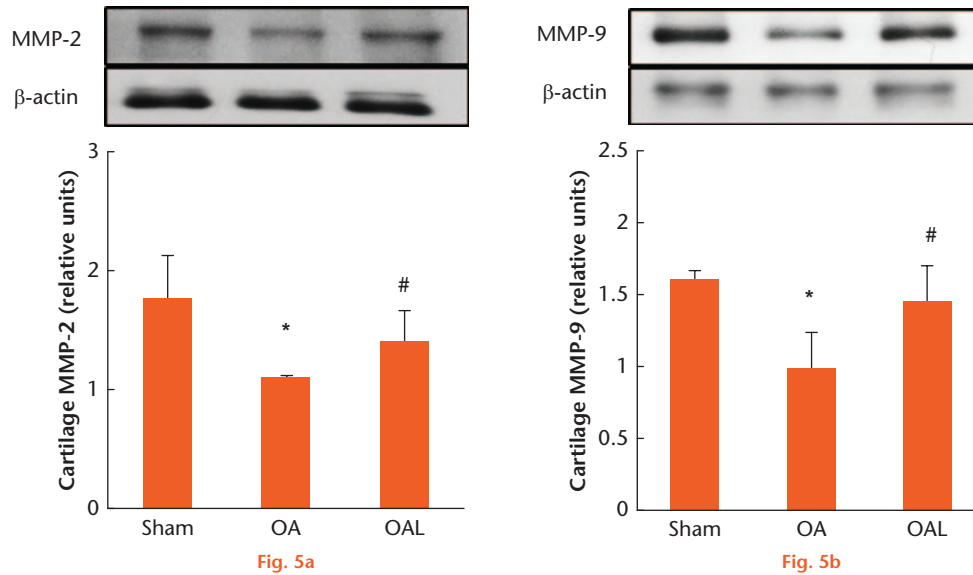
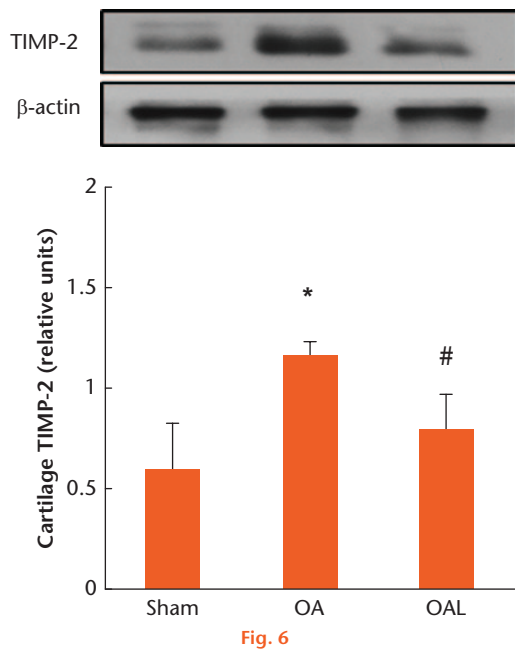


Fig. 4c

The role on nitric oxide on cartilage pro-inflammatory cytokine production in rats. Rats were divided into three groups of five. Group I (sham group), rats received the sham operation only; Group II (OA group), rats received the meniscectomy operation only; and Group III (OAL group), rats received the meniscectomy operation and L-NAME (20 mg/kg/d for 14 days, orally). Cartilage pro-inflammatory cytokines including TNF- $\alpha$  (A), IL-1 $\beta$  (B), and IL-6 (C) were assessed 14 days after meniscectomy from each group, respectively. Data are expressed as means and standard deviation. \* $p < 0.05$  compared with Sham group (Group I). # $p < 0.05$  compared with OA group (Group II).



The role on nitric oxide on cartilage MMPs levels in rats. Rats were divided into three groups of five. Group I (sham group), rats received the sham operation only; Group II (OA group), rats received the meniscectomy operation only; and Group III (OAL group), rats received the meniscectomy operation and L-NAME (20 mg/kg/d for 14 days, orally). Cartilage levels of MMP-2 (A) and MMP-9 (B) were assessed 14 days after meniscectomy from each group, respectively. Data are expressed as means and standard deviation. \* $p < 0.05$  compared with Sham group (Group I). # $p < 0.05$  compared with OA group (Group II).



The role on nitric oxide on cartilage TIMP-2 expression in rats. Rats were divided into three groups of five. Group I (sham group), rats received the sham operation only; Group II (OA group), rats received the meniscectomy operation only; and Group III (OAL group), rats received the meniscectomy operation and L-NAME (20 mg/kg/d for 14 days, orally). The cartilage level of TIMP2 expression was assessed 14 days after meniscectomy from each group, respectively. Data are expressed as means and standard deviation. \* $p < 0.05$  compared with the sham group (Group I). # $p < 0.05$  compared with OA group (Group II).

proved disappointing.<sup>31</sup> Therefore, the maintaining of immune suppression may be beneficial in confronting cartilage contact stress during the initiation of OA. In the present study, L-NAME decreased the accumulation of

NO in cartilage and relieved the suppression of the innate immune response. Therefore, NO may play a protective role against cartilage breakdown in initiating rat OA.

Cartilage may be protected by NO regulating the balance between MMP and TIMP, both of which are involved in the pathogenesis of cartilage breakdown.<sup>32,33</sup> A previous study indicated that NO decreases MMP-2 and increases TIMP-2 expression.<sup>33</sup> In the present study, increased accumulation of NO accompanied with decreased MMPs and increased TIMP-2 expression were observed in cartilage after meniscectomy. Inhibiting NO production by L-NAME reversed the changes of MMPs and TIMP-2 expression. This evidence proves our hypothesis that NO plays a protective role against cartilage breakdown in the early stage of OA. However, further study is required to confirm this. In conclusion, NO-dependent innate immunosuppression protects cartilage from damage in the early stage of OA initiation in rats.

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#### Author Contribution

- C-C. Hsu: Study design, data acquisition, analysis, interpretation, drafting and critically revising the paper
- C-L. Lin: Study design, critically revising the paper.
- I-M. Jou: Study design, data acquisition, analysis, interpretation, critically revising the paper.
- P-H. Wang: Data acquisition.
- J-S. Lee: Study design, data acquisition, analysis, interpretation, drafting and critically revising the paper.

#### ICMJE Conflicts of Interest

- None declared

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